

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
REQUEST FOR FILING APPLICATION UNDER 37 CFR 53(b)  
WITHOUT FILING FEE OR EXECUTED INVENTOR'S DECLARATION

Assistant Commissioner for Patents  
Washington, D.C. 20231

Atty. Dkt. 510-125  
Date: **March 26, 1999**



03/26/99

This is a request for filing a new PATENT APPLICATION under Rule 53(b) entitled:

**AN ORPHAN NUCLEAR RECEPTOR**

without a filing fee and/or without an executed inventor's oath/declaration.

This application is made by the below identified inventor(s). Attached hereto are the following papers:

- ☒ An abstract together with  
48 pages of specification and claims including  
24 numbered claims and also attached is/are  
12 sheets of accompanying drawings.  
☐ This application is based on the following prior foreign application(s):

Application No.	Country	Filing Date
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respectively, and priority is hereby claimed therefrom.

- ☒ This application is based on the following prior provisional application(s):

Application No.	Filing Date
60/079,593	27 March 1998

respectively, and priority is hereby claimed therefrom.

☐ Certified copy/ies of foreign applications attached.

This application is a ☐ continuation/☐ division/☐ continuation-in-part of application Serial No. , filed

Please amend the specification by inserting before the first line: --This application is a ☐ continuation/☐ division/☐ continuation-in-part of application Serial No. , filed --

Please amend the specification by inserting before the first line: --This is a continuation of PCT application No. , filed --

Please amend the specification by inserting before the first line: --This application claims the benefit of U.S. Provisional Application No. **60/079,593**, filed **27 March 1998**.--

Preliminary amendment to claims (attached hereto), to be entered before calculation of the fee.

Also attached. SEQUENCE LISTING (paper and computer readable copies)

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MJW:tat

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☒ Also attached. SEQUENCE LISTING (paper and computer readable copies)

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By Atty.: **Mary J. Wilson**, Reg. No. **32,955**

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Our Ref.: 510-125

# ***U.S. PATENT APPLICATION***

*Inventor(s):* Steven A. Kliewer  
Stacey A. Jones  
Timothy M. Willson

*Invention:* AN ORPHAN NUCLEAR RECEPTOR

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## ***SPECIFICATION***

## AN ORPHAN NUCLEAR RECEPTOR

The present application claims priority from Provisional Application No. 60/079,593, filed March 27, 1998, the entire contents of that provisional application being incorporated herein by reference.

### TECHNICAL FIELD

The present invention relates to a novel human orphan nuclear receptor that binds to a cytochrome P-450 monooxygenase (CYP) promoter and that is activated by compounds that induce CYP gene expression. The invention further relates to nucleic acid sequences encoding such a receptor, to methods of making the receptor and to methods of using the receptor and nucleic acid sequences encoding same. The invention also relates to non-human animals transformed to express the human receptor and to methods of using such animals to screen compounds for drug interactions and toxicities.

### BACKGROUND OF THE INVENTION

Members of the cytochrome P-450 (CYP) family of hemoproteins are critical in the oxidative metabolism of a wide variety of endogenous substances and xenobiotics, including various carcinogens and toxins (Nebert et al, Ann. Rev. Biochem. 56:945-993 (1987)). In man, the CYP3A4 monooxygenase plays a major role in the biotransformation of drugs due to its abundance in liver and intestine and its broad substrate

specificity. CYP3A4 catalyzes the metabolism of  
>60% of all drugs that are in use including  
steroids, immunosuppressive agents, imidazole  
antimycotics, and macrolide antibiotics (Maurel, P.  
5 in Cytochromes P450: metabolic and toxicological  
aspects (ed. Ioannides, C.) 241-270 (CRC Press,  
Inc., Boca Raton, FL, 1996).

Expression of the CYP3A4 gene is markedly  
induced both *in vivo* and in primary hepatocytes in  
10 response to treatment with a variety of compounds.  
Many of the most efficacious inducers of CYP3A4  
expression are commonly used drugs such as the  
glucocorticoid dexamethasone, the antibiotic  
rifampicin, the antimycotic clotrimazole, and the  
15 hypocholesterolemic agent lovastatin (Maurel, P. in  
Cytochromes P450: metabolic and toxicological  
aspects (ed. Ioannides, C.) 241-270 (CRC Press,  
Inc., Boca Raton, FL, 1996), Guzelian, P.S. in  
Microsomes and Drug Oxidations (eds. Miners, J.O.,  
20 Birkett, D.J., Drew, R. & McManus, M.) 148-155  
(Taylor and Francis, London, 1988). The  
inducibility of CYP3A4 expression levels coupled  
with the broad substrate specificity of the CYP3A4  
protein represent the basis for many drug  
25 interactions in patients undergoing combination drug  
therapy. While attempts have been made to develop  
*in vivo* and *in vitro* assays with which to profile  
the effects of compounds on CYP3A expression levels,  
these efforts have been hampered by species-specific  
30 effects that have limited the utility of using  
animals and their tissues for testing purposes.  
Thus, analysis of the effects of new compounds on

CYP3A4 gene expression has been largely restricted to laborious assays involving human liver tissue.

Recently, efforts have been directed at understanding the molecular basis for the induction of CYP3A4 gene expression. The CYP3A4 promoter has been cloned and a 20 bp region residing approximately 150 bp upstream of the transcription initiation site shown to confer responsiveness to dexamethasone and rifampicin (Hashimoto et al, Eur. J. Biochem. 218:585-595 (1993), Barwick et al, Molec. Pharmacol. 50:10-16 (1996)). This region contains two copies of the AG(G/T)TCA motif recognized by members of the nuclear receptor superfamily, suggesting that a nuclear receptor might be responsible for mediating at least some of the effects of the chemical inducers of CYP3A4 expression. However, prior to the present invention, proteins that bind to this response element had not been characterized.

The present invention is based on the identification of a novel orphan nuclear receptor that binds to a response element in the CYP3A4 promoter and that is activated by a range of compounds known to induce CYP3A4 expression. The identification of this receptor makes possible assays that can be used to establish whether drugs will interact *in vivo*.

#### SUMMARY OF THE INVENTION

The present invention relates to a novel human orphan nuclear receptor, designated the human pregnane X receptor (hPXR), that binds to a CYP

promoter, for example, the rifampicin/dexamethasone response element in the cytochrome P-450 monooxygenase 3A4 (CYP3A4) promoter. The receptor is activated to modulate transcription of a CYP (e.g., CYP3A4) gene. The present invention further relates to nucleic acids encoding hPXR, including expression vectors that can be used to effect expression of the receptor in host cells. The invention also relates to host cells transformed with such expression vectors and to methods of using the receptor and receptor encoding sequences in assays designed to screen compounds (e.g., drugs) for their ability to modulate CYP (e.g., CYP3A4) gene expression. The invention also relates to non-human animals transformed to express the human receptor and to methods of using same in drug screens.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D. Molecular cloning of hPXR.

(Fig. 1 A) Nucleotide (SEQ ID NO:13) and predicted amino acid (SEQ ID NO:14) sequences of hPXR.

(Fig. 1B) Amino acid sequence comparison between hPXR, mPXR1, Xenopus orphan nuclear receptor 1 (xONR1) (Smith et al, Nucl. Acids Res. 22:66-71 (1994)), and the human vitamin D receptor (hVDR). Numbers indicate percent amino acid identity in the DBDs and LBDs. (Fig. 1C) The hPXR clone encodes a functional nuclear receptor. Transfection assays were performed with a pSG5-hPXR expression vector containing the wild-type 5' region of the hPXR cDNA and a reporter plasmid containing four copies of the



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(-) or presence (+) of the pSG5-hPXR ATG expression  
plasmid and treated with vehicle alone (open bars)  
or 10  $\mu$ M dexamethasone-t-butylacetate (closed bars).  
Cell extracts were subsequently assayed for CAT  
5 activity. Data represent the mean of assays  
performed in triplicate  $\pm$  S.E. (Fig. 3B)  
Oligonucleotides used in band shift assays. The  
positions of nuclear receptor half-site motifs and  
mutations are indicated. (Fig. 3C) Band shift  
10 assays were performed with a radiolabeled  
oligonucleotide containing the CYP3A4 IR6 PXRE and  
hRXR and either hPXR (top panel) or mPXR1 (bottom  
panel). Unlabeled competitor oligonucleotides were  
added at a 10-fold or 50-fold molar excess as  
15 indicated.

Figures 4A-4C. hPXR is activated by  
structurally-distinct inducers of CYP3A4 gene  
expression. (Fig. 4A) CV-1 cells were transfected  
with the pSG5-hPXR ATG or pSG5-mPXR1 expression  
20 plasmids and the (IR6)<sub>3</sub>-tk-CAT reporter (left and  
middle panels, respectively), or the RS-hGR  
expression plasmid (Giguere et al, Cell 46:645-652  
(1986)) and a reporter containing two copies of a  
consensus glucocorticoid response element upstream  
25 of tk-CAT (right panel). Cells were treated with  
1  $\mu$ M mevastatin or lovastatin, 100  $\mu$ M phenobarbital,  
or 10  $\mu$ M of the other compounds. Cell extracts were  
subsequently assayed for CAT activity. Data  
represent the mean of assays performed in triplicate  
30  $\pm$  S.E. (Fig. 4B) Structures of representative  
compounds that activate hPXR. (Fig. 4C) CARLA was  
performed with bacterially-expressed GST-hPXR or

5 GST-mPXR1 and [<sup>35</sup>S]SRC1.14 synthesized *in vitro*.  
[<sup>35</sup>S]SRC1.14 was mixed with either GST-hPXR or GST-  
mPXR1 in the presence of vehicle alone (1) (1% DMSO)  
or 10 μM of dexamethasone-t-butylacetate (2),  
rifampicin (3), or clotrimazole (4). [<sup>35</sup>S]SRC1.14  
complexed with GST-hPXR (top panel) or GST-mPXR1  
(bottom panel) was precipitated with glutathione-  
sepharose beads.

10 Figure 5. Reaction scheme for production of  
[<sup>3</sup>H]GW-485801.

Figure 6. Plot of specific binding vs.  
concentration of [<sup>3</sup>H]GW-485801. K<sub>d</sub> = 370 nM.

#### DETAILED DESCRIPTION OF THE INVENTION

15 The present invention relates to a novel human  
nuclear receptor, hPXR. The invention further  
relates to nucleic acid sequences encoding hPXR, to  
constructs comprising such sequences, to host cells  
containing the constructs and to a method of  
producing hPXR using such host cells. The invention  
20 also relates to non-human animals transformed to  
express hPXR. The invention further relates to *in*  
*vivo* and *in vitro* assays that can be used to  
identify compounds that induce CYP expression.  
While the disclosure that follows makes specific  
25 reference to CYP3A4, it should be appreciated that  
the details (e.g., methods) provided find  
application in connection with other CYP genes as  
well.

hPXR is characterized as a protein comprising about 434 amino acids and having a molecular weight of about 49.7 kilodaltons. hPXR binds to a DNA response element in the CYP3A4 promoter as a heterodimer with the 9-cis retinoic acid receptor, RXR. hPXR is activated by compounds known to modulate CYP3A4 expression. The receptor is most abundantly expressed in liver but is also present in colon and small intestine.

One embodiment of the receptor of the invention has the amino acid sequence set forth in Figure 1, or an analog thereof (wherein the term analog is intended to indicate a naturally occurring human variant of the Figure 1 sequence), or a fragment thereof, including fragments having at least one functional characteristic of hPXR (e.g., ligand binding or DNA binding). Preferred fragments include portions of the Figure 1 sequence at least 30 consecutive amino acids in length, more preferably, at least 50 consecutive amino acids in length, and most preferably, at least 75 consecutive amino acids in length. Specific fragments include the ligand binding domain (that is, amino acids 141 to 434 of the Figure 1 sequence) and the DNA binding domain (that is, amino acids 41 to 107 of the Figure 1 sequence) as well as the domain that is used for the ligand binding assay described in the Examples that follow (that is, amino acids 130-434 of the Figure 1 sequence). The invention also includes a protein comprising a domain sharing at least 80% amino acid sequence identity with the ligand binding domain of the Figure 1 sequence, more preferably, at

least 85% amino acid sequence identity and, most preferably, at least 90% or 95%, 96%, 97%, 98% or 99% amino acid sequence identity with the ligand binding domain of the Figure 1 sequence (% sequence identity being determined, for example, by Basic Blast (version 2.0) available through the NCBI website <http://www.ncbi.nlm.nih.gov/>), and, advantageously, retaining the function of the Figure 1 sequence.

The receptor of the invention, or fragment thereof, can bear a detectable label (e.g., a radioactive or fluorescent label). The receptor, or receptor fragment, can also be bound to a solid support, e.g., a glass or plastic particle, a plate, or a filter.

Nucleic acid sequences of the invention include DNA and RNA sequences encoding hPXR, for example, hPXR having the amino acid sequence given in Figure 1, as well as nucleic acid sequences encoding analogs and fragments of the Figure 1 amino acid sequence as defined above, and nucleic acid sequences encoding proteins comprising a domain sharing at least 80% amino acid sequence identity (more preferably, at least 85%, or at least 90%, or at least 95%, or at least 96%, or at least 97%, or at least 98% or at least 99%) with the ligand binding domain of the Figure 1 sequence, as described above. A specific nucleic acid sequence of the invention is that shown in Figure 1.

The hPXR encoding sequence can be present in a construct, for example, in an expression construct, operably linked to a promoter (e.g., the CMV, SV40,

Taq, T7 or LacO promoter). Such expression constructs are operative in a cell in culture (e.g., yeast, bacteria, insect or mammalian), to express the encoded hPXR, or fragment thereof. Preferred expression vectors include pGEX, pET, pFASTbacHT and pSG5.

The invention also relates to cells in culture (e.g., yeast, bacteria or mammalian (for example, CV-1, HuH7, HepG2, or CaCo2 cells)) that are transformed with an above-described construct. Transformation can be effected using any of a variety of standard techniques. Such cells can be used in a method of making hPXR (or fragment thereof) by culturing same under conditions suitable for expression of the polypeptide product.

The invention further relates to chimeric receptors (or fusion proteins having a receptor component) (and encoding sequences) comprising at least a DNA-binding domain or a ligand-binding domain of hPXR, and a non-hPXR derived sequence. Non-hPXR derived sequences can be selected so as to be suitable for the purpose to be served by the chimeric receptor. Examples of such sequences include glutathione-S-transferase and the DNA binding domain of yeast transcription factor GAL4 and other DNA binding domains, e.g., DNA binding domains for the estrogen and glucocorticoid receptors. The chimeric receptor can bear a detectable label (e.g., a radioactive or fluorescent label). The chimeric receptor can also be bound to a solid support, e.g., a glass or plastic particle, a plate or a filter.

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A further aspect of the invention relates to *in vitro* (cell-free) and *in vivo* (cell-based) assays that can be used to profile the effects of compounds (e.g. potential new drugs) on CYP3A4 levels. The inducibility of CYP3A4 levels, coupled with the broad substrate specificity of the CYP3A4 enzyme, represent the basis for many drug-drug interactions in patients undergoing multiple drug therapy. Ideally, new drugs would have little or no effect on CYP3A4 expression levels.

The assays of the invention can take any of a variety of forms. As compounds that activate hPXR function as inducers of CYP3A4 gene expression, hPXR binding and activation assays provide efficient means to identify compounds that can be expected to activate CYP3A4.

Binding assays of the invention include cell free assays in which hPXR, or the ligand binding domain thereof (alone or present as a fusion protein), is incubated with a test compound which, advantageously, bears a detectable label (e.g., a radioactive or fluorescent label). The hPXR, or ligand binding domain thereof, free or bound to test compound, is then separated from free test compound using any of a variety of techniques (e.g., using gel filtration chromatography (for example, on Sephadex G50 spin columns) or through capture on a hydroxyapatite resin). The amount of test compound bound to hPXR or ligand binding domain thereof, is then determined (for example, by liquid scintillation counting in the case of radiolabelled test compounds).

An alternative approach for detecting radiolabeled test compound bound to hPXR, or ligand binding domain thereof, is a scintillation proximity assay (SPA). In this assay, a bead (or other  
5 particle) is impregnated with scintillant and coated with a molecule that can capture the hPXR, or ligand binding domain thereof (e.g., streptavidin-coated beads can be used to capture biotinylated hPXR ligand binding domain). Radioactive counts are  
10 detected only when the complex of radiolabeled test compound and the hPXR, or ligand binding domain thereof, is captured on the surface of the SPA bead, bringing the radioactive label into sufficient proximity to the scintillant to emit a signal. This  
15 approach has the advantage of not requiring the separation of free test compound from bound (Nichols et al, Anal. Biochem. 257:112-119 (1998)).

Assays to determine whether a test compound interacts with the hPXR ligand binding domain can  
20 also be performed via a competition binding assay. In this assay, hPXR, or ligand binding domain thereof, is incubated with a compound known to interact with hPXR, which compound, advantageously, bears a detectable label (e.g., a radioactive or  
25 fluorescent label (see Example 5 - Crabtree catalysts suitable for use in the synthetic approach described in Example 5 include those reported by Chen et al, J. Labelled Compd. Radiopharm. 39:291 (1997) and Crabtree et al, Inorg. Synth. 28:56  
30 (1990))). A test compound is added to the reaction and assayed for its ability to compete with the labeled compound for binding to hPXR, or ligand

binding domain thereof. A standard assay format employing a step to separate free known (labeled) compound from bound, or an SPA format, can be used to assess the ability of the test compound to  
5 compete.

A further example of a binding assay in accordance with the invention is based on the finding that hPXR ligands induce the interaction of hPXR ligand binding domain with coactivators (e.g.,  
10 SRC1, TIF-1, TIF-2 or ACTR, or fragment thereof). To determine if a test compound activates hPXR, and thus induces CYP3A4 expression, the ligand binding domain of hPXR is prepared (e.g., expressed) as a fusion protein (e.g., with glutathione-S-transferase  
15 (GST), a histidine tag or a maltose binding protein). The fusion protein and coactivator (either or both advantageously labeled with a detectable label, e.g., a radiolabel or fluorescent tag) are incubated in the presence and absence of  
20 the test compound and the extent of binding of the coactivator to the fusion protein determined. The induction of interaction in the presence of the test compound is indicative of an hPXR activator.

hPXR activation assays in accordance with the  
25 invention can be carried out using full length hPXR and a reporter system comprising one or more copies of the DNA binding site recognized by the hPXR binding domain (see Example 3). Advantageously, however, the activation assays are conducted using  
30 established chimeric receptor systems. For example, the ligand binding domain of hPXR can be fused to the DNA binding domain of, for example, yeast

transcription factor GAL4, or that of the estrogen or glucocorticoid receptor. An expression vector for the chimera (e.g., the GAL4-hPXR chimera) can be transfected into host cells (e.g., CV-1, HuH7, HepG2 or CaCo2 cells) together with a reporter construct. The reporter construct can comprise one or more (e.g., 5) copies of the DNA binding site recognized by the binding domain present in the chimera (e.g., the GAL4 DNA binding site) driving expression of a reporter gene (e.g., CAT, SPAP or luciferase). Cells containing the constructs are then treated with either vehicle alone or vehicle containing test compound, and the level of expression of the reporter gene determined. In accordance with this assay, enhancement of expression of the reporter gene in the presence of the test compound indicates that the test compound activates hPXR and thus can function as an inducer of CYP3A4 gene expression. (See Example 4.)

Another format suitable for use in connection with the present invention is the yeast two-hybrid assay. This is an established approach to detect protein-protein interactions that is performed in yeast. Protein #1, representing the bait, is expressed in yeast as a chimera with a DNA binding domain (e.g., GAL4). Protein #2, representing the predator, is expressed in the same yeast cell as a chimera with a strong transcriptional activation domain. The interaction of bait and predator results in the activation of a reporter gene (e.g., luciferase or  $\beta$ -galactosidase) or the regulation of a selectable marker (e.g., LEU2 gene). This

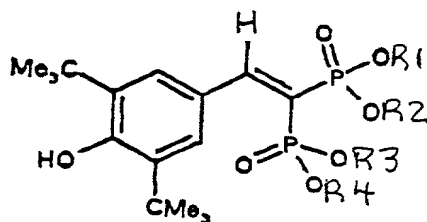
approach can be used as a screen to detect, for example, ligand-dependent interactions between hPXR1 and other proteins such as coactivator proteins (e.g., SRC1, TIF1, TIF2, ACTR) or fragments thereof.  
5 (Fields et al, Nature 340:245-246 (1989)).

Still another format is the ligand-induced complex formation (LIC) assay. This is an approach to detect ligand-mediated effects on nuclear receptor-DNA interactions. hPXR (or, minimally, the  
10 DNA and ligand binding domains thereof) can be incubated with its heterodimeric partner RXR in the presence of DNA representing an established hPXR/RXR binding site. Test compounds can be assayed for their ability to either enhance or interfere with  
15 binding of the hPXR/RXR heterodimer to DNA (Forman et al, Proc. Natl. Acad. Sci. USA 94:4312-4317 (1997)).

Compounds that bind PXR with a suitable  $pK_i$ , for example with a  $pK_i > 5$ , can be screened for  
20 selectivity for PXR versus other nuclear receptors (e.g., RXR) using standard binding assays. A compound that binds selectively to PXR (that is, has at least a 10 fold greater affinity for PXR, preferably, at least a 100 fold greater affinity for  
25 PXR, than, for example, the glucocorticoid receptor) and thereby affects the functional activity of PXR in a cell (e.g., a cell in culture, a cell present in a tissue or a cell present in a whole animal) can be used to associate PXR activity with a mammalian  
30 disease state. For example, a compound that activates PXR induces CYP3A. Thus, diseases in which CYP3A activity is important are associated

with PXR, and compounds that activate or deactivate PXR may be useful in prevention or treatment of such diseases. By using the associating methods of this invention, new PXR-associated diseases can be  
5 discovered. Once these new associations are discovered, new drugs for these diseases can be identified by screening for compounds that activate or deactivate PXR.

10 An example of a compound suitable for use in making disease associations in accordance with the method described above is the compound of formula I:



wherein each of R1, R2, R3 and R4 is,  
independently, C<sub>1</sub>-C<sub>6</sub>alkyl (linear or branched),  
preferably, C<sub>2</sub> or C<sub>3</sub>alkyl (e.g., ethyl, n-propyl or  
iso-propyl), more preferably, C<sub>2</sub>alkyl. The compounds  
5 can be labelled with a detectable label, e.g., a  
radiolabel, e.g., tritium.

Another aspect of the invention relates to  
transgenic animals that express hPXR. For example,  
transgenic mice can be generated that express the  
10 hPXR gene as well as the endogenous mouse PXR gene.  
Mice can also be generated in which the endogenous  
PXR gene is knocked out and then replaced by the  
hPXR gene. Transgenic animals can be generated that  
express isoforms of hPXR as well as mutant alleles  
15 of the gene. Transgenic animals developed by these  
methods can be used to screen compounds for drug  
interactions and toxicities, and to study the  
regulation of CYP3A in vivo.

A further aspect of the present invention  
20 relates to diagnostic assays that can be used to  
screen for mutations in hPXR that alter the ability  
of the receptor to induce CYP3A4 gene expression.  
These assays can be based on the sequencing of the  
hPXR gene, on hybridization approaches designed to  
25 detect sequence changes or polymorphisms, or the use  
of antibodies to distinguish wild-type from  
mutant/polymorphic hPXR. Changes that result in  
alteration of the DNA binding or ligand binding  
characteristics of hPXR can be expected to have a  
30 significant impact on hPXR activity. A mutation or  
polymorphism in hPXR can be indicative of a patient  
at increased risk of suffering an adverse reaction

to a drug as a result of unusual rates of drug metabolism.

The invention also relates to antibodies, polyclonal or monoclonal, that are specific for hPXR, and antigen binding fragments thereof (e.g., Fab fragments). The antibodies can be generated in accordance with standard techniques using intact hPXR or a fragment thereof as defined above. The antibodies can be used, for example, in assays to detect the presence of the receptor. Further, the antibodies can be used in hPXR purification protocols.

The invention also relates to kits suitable for use, for example, in one or more method described above. The kits can include hPXR (or fragment thereof) or nucleic acid encoding same or antibodies as described above. The kit can also include compounds that bind hPXR, such as GW-485801. The hPXR, nucleic acid and/or antibody can be present in the kit disposed within a container means. The kit can also include ancillary reagents and buffers, etc., to facilitate practice of the specific method.

Certain aspects of the present invention are described in greater detail in the non-limiting Examples that follow.

#### EXAMPLES

The following experimental details are relevant to the specific Examples that follow.

### Chemicals

Dexamethasone-t-butylacetate and RU486 were purchased from Research Plus, Inc. (Bayonne, NJ) and Biomol (Plymouth Meeting, PA), respectively. All other compounds were purchased from either Sigma Chemical Co. (St. Louis, MO) or Steraloids, Inc. (Wilton, NH).

### Molecular cloning of hPXR cDNAs

An EST was identified in the Incyte database (clone identification number 2211526) that contained nucleotides 444-2111 of the hPXR sequence. An oligonucleotide derived from this EST sequence (5' CTGCTGCGCATCCAGGACAT 3') (SEQ ID NO:1) was used to screen a pCMV-SPORT human liver cDNA library (Gibco/BRL) using Gene Trapper solution hybridization cloning technology (Gibco/BRL). Two clones were obtained that encoded hPXR, one containing nucleotides 1-2125, the other containing nucleotides 102-2118. The sequence of the longer is shown in Figure 1A. Sequences were aligned and analyzed by the University of Wisconsin Genetics Computer Group programs.

### Plasmids

The expression vector pSG5-hPXR was generated by PCR amplification and subcloning of nucleotides 1-1608 of the hPXR clone into the pSG5 expression vector (Stratagene). pSG5-hPXR ATG was generated by PCR amplification of cDNA encoding amino acids 1-434 of hPXR using oligonucleotides 5'-GGGTGTGGGGAATCCACCACCATGGAGGTGAGACCCAAAGAAAGC-3' (SEQ ID NO:2) (sense) and 5'-

GGGTGTGGGGGATCCTCAGCTACCTGTGATGCCG-3' (SEQ ID NO:3)  
 (antisense) and insertion into EcoRI/BamHI-cut pSG5.  
 The bacterial expression vector pGEX-hPXR was  
 generated by PCR amplification of cDNA encoding  
 5 amino acids 108-434 and insertion into pGEX-2T  
 (Pharmacia). The reporter plasmid (DR3)<sub>4</sub>-tk-CAT was  
 generated by insertion of four copies of a double-  
 stranded oligonucleotide containing the CYP3A1 DR3  
 PXRE (5'-GATCAGACAGTTCATGAAGTTCATCTAGATC-3') (SEQ ID  
 10 NO:4) into the BamHI site of pBLCAT2 (Luckow et al,  
 Nucl. Acids Res. 15:5490 (1987)). The reporter  
 plasmid (IR6)<sub>3</sub>-tk-CAT was generated by insertion of  
 three copies of the CYP3A4 IR6 PXRE (5'-  
 GATCAATATGAACTCAAAGGAGGTCAGTG-3') (SEQ ID NO:5) into  
 15 the BamHI site of pBL2CAT. The pRSET-SRC1.14  
 expression plasmid has been previously described  
 (Kliwer, S.A., et al. Cell 92:73-82 (1998)). All  
 constructs were confirmed by sequence analysis.

#### Cotransfection assays

20 CV-1 cells were plated in 24-well plates in DME  
 medium supplemented with 10% charcoal-stripped fetal  
 calf serum at a density of  $1.2 \times 10^5$  cells per well.  
 In general, transfection mixes contained 33 ng of  
 receptor expression vector, 100 ng of reporter  
 25 plasmid, 200 ng of  $\beta$ -galactosidase expression vector  
 (pCH110, Pharmacia), and 166 ng of carrier plasmid.  
 Cells were transfected overnight by lipofection  
 using Lipofectamine (Life Technologies, Inc.),  
 according to the manufacturer's instructions. The  
 30 medium was changed to DME medium supplemented with  
 10% delipidated calf serum (Sigma) and cells were

incubated for an additional 24 hours. Cell extracts were prepared and assayed for CAT and  $\beta$ -galactosidase activities as previously described (Lehmann et al, J. Biol. Chem. 270:12953-12956 (1995)).

#### Northern analysis

An approximately 1.0 kb fragment encoding the LBD of hPXR was [<sup>32</sup>P]-labeled by random priming and used to probe mouse multiple tissue Northern blots (Clontech). Blots were hybridized in ExpressHyb solution (Clontech) at 42°C overnight. Final washes were performed with 0.1x SSC, 0.1% SDS at 58°C.

#### Band shift assays

hPXR, mPXR1, and hRXR were synthesized *in vitro* using the TNT rabbit reticulocyte lysate coupled *in vitro* transcription/translation system (Promega) according to the manufacturer's instructions. Gel mobility shift assays (20  $\mu$ l) contained 10 mM Tris (pH 8.0), 40 mM KCl, 0.05% NP-40, 6% glycerol, 1 mM DTT, 0.2  $\mu$ g of poly(dI-dC) and 2.5  $\mu$ l each of *in vitro* synthesized PXR and RXR proteins. Competitor oligonucleotides were included at a 10-fold or 50-fold excess. After a 10 min incubation on ice, 10 ng of [<sup>32</sup>P]-labeled oligonucleotide was added and the incubation continued for an additional 10 min. DNA-protein complexes were resolved on a 4% polyacrylamide gel in 0.5X TBE (1X TBE = 90 mM Tris, 90 mM boric acid, 2 mM EDTA). Gels were dried and subjected to autoradiography at -70°C. The following

oligonucleotides were used as either radiolabeled probes or competitors (sense strand is shown):

CYP3A4 IR6: 5' GATCAATATGAACTCAAAGGAGGTCAAGT 3'  
(SEQ ID NO:6)

5 CYP3A4 IR6m1 5' GATCAATATGTTCTCAAAGGAGAACAGT 3'  
(SEQ ID NO:7)

CYP3A4 IR6m2 5' GATCAATAACAACTCAAAGGAGGTCAAGT 3'  
(SEQ ID NO:8)

CYP3A1 DR3: 5' GATGCAGACAGTTCATGAAGTTCATCTAGATC  
10 3' (SEQ ID NO:9).

#### CARLA

GST-hPXR fusion protein was expressed in BL21(DE3)plysS cells and bacterial extracts prepared by one cycle of freeze-thaw of the cells in Protein  
15 Lysis Buffer containing 10 mM Tris, pH 8.0, 50 mM KCl, 10 mM DTT, and 1% NP-40 followed by centrifugation at 40,000 x g for 30 minutes. Glycerol was added to the resulting supernatant to a final concentration of 10%. Lysates were stored at  
20 -80°C. [<sup>35</sup>S]SRC1.14 was generated using the TNT rabbit reticulocyte system (Promega) in the presence of Pro-Mix (Amersham). Coprecipitation reactions included 25 µl of lysate containing GST-hPXR fusion protein, 25 µl Incubation Buffer (50 mM KCl, 40 mM  
25 HEPES pH 7.5, 5 mM β-mercaptoethanol, 1% Tween-20, 1% non-fat dry milk), 5 µl [<sup>35</sup>S]SRC1.14, and vehicle (1% DMSO) or compounds as indicated. The mixtures were incubated for 25 minutes at 4°C with gentle mixing prior to the addition of 15 µl of  
30 glutathione-sepharose 4B beads (Pharmacia) that had been extensively washed with Protein Lysis Buffer. Reactions were incubated with gentle mixing at 4°C

for an additional 25 min. The beads were pelleted at 3000 rpm in a microfuge and washed 3 times with Protein Incubation Buffer containing either vehicle alone, dexamethasone-t-butylacetate, rifampicin, or clotrimazole. After the last wash, the beads were resuspended in 25 µl of 2X SDS-PAGE sample buffer containing 50 mM DTT. Samples were heated at 100°C for 5 minutes and loaded onto a 10% Bis-Tris PAGE gel. Gels were dried and subjected to autoradiography.

#### EXAMPLE 1

##### Molecular Cloning and Tissue Expression Pattern of hPXR

A human EST was identified in the Incyte LifeSeq® proprietary database that was highly homologous to a region of mPXR1 (Kliewer et al, Cell 92:73-82 (1998)). Two larger clones were isolated in a screen of a human liver cDNA library using an oligonucleotide within the EST as a probe. The longest of these clones was 2146 bp in length (Fig. 1A) and encoded a new member of the nuclear receptor superfamily that was 97% and 76% identical to mPXR1 in the DNA binding domain (DBD) and ligand binding domain (LBD), respectively (Fig. 1B). In terms of other members of the nuclear receptor superfamily, hPXR was most closely related to the *Xenopus laevis* orphan receptor ONR1 (Smith et al, Nucl. Acids Res. 22:66-71 (1994)) and the vitamin D receptor (Fig. 1B). Notably, the hPXR sequence lacked an AUG initiator codon in between an in-frame stop codon (nucleotides 205-207 in the hPXR

sequence) and the start of the region encoding the DBD. However, transfection experiments performed in CV-1 cells with the hPXR clone and a reporter plasmid containing four copies of an established mPXR binding site from the rat CYP3A1 gene promoter inserted upstream of the minimal thymidine kinase (tk) promoter and the chloramphenicol acetyltransferase (CAT) gene (Kliewer et al, Cell 92:73-82 (1998)) demonstrated that the hPXR clone encoded a functional nuclear receptor that was activated efficiently by dexamethasone-t-butylacetate, a known mPXR1 ligand (Kliewer et al, Cell 92:73-82 (1998)) (Fig. 1C).

Examination of the hPXR sequence revealed an in-frame CUG codon (nucleotides 304-306) surrounded by a favorable Kozak sequence (Kozak, J. Biol. Chem. 266:19867-19870 (1991)). There is precedent for the use of CUG codons to initiate translation of eukaryotic proteins, including the nuclear receptor RAR $\beta$ 4 (Kozak, J. Biol. Chem. 266:19867-19870 (1991), Nagpal et al, Proc. Natl. Acad. Sci. USA 89:2718-2722 (1992)). Initiation of translation at this CUG codon would yield a protein of 434 amino acids, three longer than mPXR1, with a predicted MW of 49.7 kD. In order to determine whether translation of the hPXR cDNA initiated at the CUG codon, hPXR RNA containing the wild-type 5' region was translated in the presence of [<sup>35</sup>S]methionine using rabbit reticulocyte lysates. As a control, hPXR RNA, in which this CUG codon had been mutated to the optimal AUG (hPXR AUG), was also translated *in vitro*. Translation of the wild-type hPXR RNA resulted in an

approximately 50 kD protein that co-migrated with the translation product of hPXR AUG RNA (Fig. 1D, open arrow with asterisk). This 50 kD product was not produced when hPXR antisense RNA was used in the translation reaction. Much lower amounts of an approximately 53 kD translation product were also produced in translation reactions performed with hPXR RNA (Fig. 1D, bent arrow), indicating that a small amount of translation initiated at other non-AUG codons upstream of the CUG codon. However, the results indicate that the CUG codon represents the principal translation initiation site for hPXR containing a functional DBD.

The tissue expression pattern of hPXR was next examined via Northern analysis using blots containing poly(A)<sup>+</sup> RNA prepared from multiple adult tissues. hPXR mRNA was expressed most abundantly in liver and was also present in the colon and small intestine (Fig. 2). Three transcripts of different size were detected in each of these tissues: a prominent 2.6 kb product and two less abundant messages of approximately 4.3 kb and 5 kb. It was recently shown that the mPXR gene is also abundantly expressed in liver and small intestine (Kliewer et al, Cell 92:73-82 (1998)). Whereas mPXR message was also detected at low levels in stomach and kidney, mRNA for hPXR was not detected in these tissues (Fig. 2). Thus, both hPXR and mPXR are most abundantly expressed in the liver and tissues of the gastrointestinal tract; however, there are differences in PXR expression patterns in mice and humans.

## EXAMPLE 2

### hPXR Activates Transcription Through a Response Element in the CYP3A4 Gene Promoter

Several lines of evidence have been provided  
5 that mPXR1 regulates CYP3A1 gene expression: mPXR1  
was activated by compounds known to activate CYP3A1  
gene expression including glucocorticoids and  
antiglucocorticoids, mPXR1 and CYP3A1 gene  
expression colocalized in the liver and small  
10 intestine, and mPXR1 bound to a response element in  
the CYP3A1 gene promoter that had previously been  
determined to confer responsiveness to  
glucocorticoids and antiglucocorticoids (Kliewer et  
al, Cell 92:73-82 (1998), Quattrochi et al, J. Biol.  
15 Chem. 270:28917-28923 (1995), Huss et al, J. Biol.  
Chem. 93:4666-4670 (1996)). The findings that the  
CYP3A4 gene is also expressed in the liver and  
intestine and that this expression is induced in  
response to glucocorticoids and antiglucocorticoids  
20 (Molawa et al, Proc. Natl. Acad. Sci. USA 83:5311-  
5315 (1986), Kocarek et al, Drug Met. Dispos.  
23:415-421 (1995)) led to the investigation of  
whether hPXR regulates CYP3A4 gene expression.

The induction of CYP3A4 expression in response  
25 to dexamethasone and rifampicin has been localized  
to an approximately 20 bp region of the promoter  
that contains two copies of the nuclear receptor  
half-site sequence AG(G/T)TCA organized as an  
inverted repeat (IR) and separated by 6 base pairs,  
30 an IR6 motif (Barwick et al, Molec. Pharmacol.  
50:10-16 (1996)) (Fig. 3B). This IR6 motif is  
highly conserved in the promoters of CYP3A gene

family members of several species (Barwick et al, Molec. Pharmacol. 50:10-16 (1996)). Interestingly, this half-site configuration is very different from that found in the CYP3A1 PXR response element (PXRE) which contains two half-sites organized as a direct repeat (DR) with a 3 nucleotide spacer, a DR3 motif (Kliewer et al, Cell 92:73-82 (1998)). To determine whether hPXR could regulate transcription through the IR6 motif, a reporter plasmid was generated containing three copies of the CYP3A4 IR6 response element upstream of the tk promoter and CAT gene. Cotransfection assays were performed with the (IR6)<sub>3</sub>-tk-CAT reporter and pSG5-hPXR ATG expression plasmids in CV-1 cells that were either treated with vehicle alone or 10  $\mu$ M dexamethasone-t-butylacetate. hPXR induced reporter levels in the presence of dexamethasone-t-butylacetate (Fig. 3A), demonstrating that hPXR can activate transcription through the CYP3A4 IR6 motif.

In order to determine whether hPXR interacted directly with the CYP3A4 IR6 response element, band shift assays were performed. Since mPXR1 binds to DNA as a heterodimer with RXR (Kliewer et al, Cell 92:73-82 (1998)), it was suspected that hPXR would require RXR for high-affinity interactions with DNA. Neither hPXR nor RXR bound to a radiolabeled oligonucleotide containing the CYP3A4 IR6 motif on their own (Fig. 3C). However, hPXR and RXR bound efficiently as a heterodimer to the IR6 PXRE. The hPXR/RXR complex was competed efficiently by unlabeled oligonucleotides encoding either the IR6 PXRE from the CYP3A4 promoter or the DR3 PXRE from

the CYP3A1 promoter that it was previously defined  
as a mPXR1/RXR binding site (Kliewer et al, Cell  
92:73-82 (1998)) (Fig. 3C). Thus, the hPXR/RXR  
heterodimer interacted efficiently with two response  
5 elements with remarkably different architecture.  
Little or no competition was seen when competitor  
oligonucleotides were used that contained mutations  
in either the 5' half-site or both half-site  
sequences of the IR6 PXRE (Fig. 3C). The same  
10 binding profile was observed when the mPXR1 was  
substituted for hPXR (Fig. 3C). It was concluded  
from these experiments that hPXR binds efficiently  
to the CYP3A4 IR6 PXRE as a heterodimer with RXR,  
and that hPXR and mPXR1 have very similar DNA  
15 binding profiles.

### EXAMPLE 3

#### Differential Activation of Human and mPXR

CYP3A4 gene expression is induced in response  
to a remarkable array of xenobiotics, including  
20 synthetic steroids (Kocarek et al, Drug Met. Dispos.  
23:415-421 (1995), Schuetz et al, J. Biol. Chem.  
259:2007-2012 (1984), Heuman et al, Mol. Pharmacol.  
21:753-760 (1982), Schulte-Hermann et al, Cancer  
Res. 48:2462-2468 (1988)), macrolide antibiotics  
25 (Wrighton et al, Biochem. 24:2171-2178 (1985)),  
antimycotics (Hostetler et al, Mol. Pharmacol.  
35:279-285 (1989)), HMG-CoA reductase inhibitors  
(statins) (Kocarek et al, Toxicol. Appl. Pharmacol.  
120:298-307 (1993), Schuetz et al, Hepatology  
30 18:1254-1262 (1993)), and phenobarbital-like

compounds (Heuman et al, Mol. Pharmacol. 21:753-760 (1982)). It was next determined whether hPXR might mediate the effects of some or all of these compounds on CYP3A4 expression. CV-1 cells were  
5 cotransfected with the pSG5-hPXR ATG expression plasmid and the (IR6)<sub>3</sub>-tk-CAT reporter plasmid, and the cells were treated with micromolar concentrations of a number of compounds that are known to induce CYP3A gene expression in humans  
10 and/or rodents. As shown in Fig. 4A, hPXR was activated by the synthetic steroids dexamethasone, dexamethasone-t-butylacetate, PCN, RU486, spironolactone, and cyproterone-acetate. Dexamethasone-t-butylacetate and RU486 were the most  
15 efficacious activators of hPXR among the synthetic steroids tested. Notably, the antibiotic rifampicin and the antimycotic clotrimazole were both efficacious activators of hPXR (Fig. 4A). The antihypercholesterolemic drug lovastatin also  
20 activated hPXR as did phenobarbital and the organochlorine pesticide transnonachlor (Fig. 4A). Thus, hPXR is activated by a remarkably diverse group of synthetic compounds that are known to induce CYP3A4 gene expression (Fig. 4B).

25 Several naturally-occurring C21 steroids were also tested on hPXR that were previously shown to activate mPXR1 (Kliwer et al, Cell 92:73-82 (1998)). Pregnenolone, progesterone, and 5  $\beta$ -pregnane-3,20-dione all activated hPXR roughly 4-  
30 fold. The 17-hydroxy derivatives of pregnenolone and progesterone were weak activators of hPXR (Fig. 4A). These natural steroids all activated hPXR in

transient transfection assays with EC<sub>50</sub> values >10  
μM, suggesting that they are unlikely to be natural  
hPXR ligands. However, related pregnanes or  
pregnane metabolites may serve as natural hPXR  
5 ligands.

Analyses of the effects of chemical inducers of  
CYP3A gene expression in primary hepatocytes  
obtained from either rodents or humans have revealed  
significant interspecies differences (Barwick et al,  
10 Molec. Pharmacol. 50:10-16 (1996), Kocarek et al,  
Drug Met. Dispos. 23:415-421 (1995)). For example,  
rifampicin is an efficacious inducer of CYP3A4 gene  
expression in human hepatocytes but has little or no  
effect on CYP3A1 levels in rat hepatocytes. In  
15 contrast, PCN has marked effects on CYP3A levels in  
rat hepatocytes but only modest effects in human  
hepatocytes. To examine whether differences in PXR  
activation profiles might account for these  
interspecies variations, the same panel of compounds  
20 was tested on mPXR1. As shown in Fig. 4A, there  
were marked differences in the response profiles of  
the mouse and human homologs of PXR. Whereas  
rifampicin was an efficacious activator of hPXR, it  
was only a weak activator of mPXR1 (Fig. 4A).  
25 Clotrimazole, lovastatin and phenobarbital were also  
more efficacious activators of hPXR than mPXR1. In  
contrast, PCN only activated hPXR approximately 3-  
fold but activated mPXR1 roughly 9-fold (Fig. 4A).  
Taken together, these data indicate that much of the  
30 interspecies variability in CYP3A regulation may be  
due to differences in PXR activation profiles.

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The panel of chemicals that induce CYP3A expression was also profiled on the human glucocorticoid receptor (GR). As shown in Fig. 4A, only dexamethasone and dexamethasone-t-butylacetate were efficacious activators of the GR. None of the other compounds activated the GR >1.5-fold (Fig. 4A). In contrast to a recent report (Calleja et al, Nature Med. 4:92-96 (1998)), activation of the GR by rifampicin was not observed. Since this previous work was performed in HepG2 cells, it may be that rifampicin is differentially metabolized in various cell lines. As expected, neither pregnenolone, progesterone, nor their 17-hydroxy derivatives had an effect on GR activity (Fig. 4A). Thus, the broad activation profile that was observed for the human and mouse homologs of PXR with inducers of CYP3A gene expression is not a general property of other steroid hormone receptors.

In the absence of high-affinity radioligands, coactivator-based assays have been used as a biochemical means to determine whether compounds that activate orphan nuclear receptors do so through direct interactions with the protein (Kliewer et al, Cell 92:73-82 (1998), Krey et al, Mol. Endocrinol. 11:779-791 (1997)). These assays are predicated on the finding that ligands induce the interaction of nuclear receptors with accessory proteins, termed coactivators (Krey et al, Mol. Endocrinol. 11:779-791 (1997)). It was recently demonstrated that several steroidal activators of mPXR1, including dexamethasone-t-butylacetate and PCN, promote the interaction of the mPXR1 LBD with a 14 kD fragment

of the steroid receptor coactivator 1 (SRC1.14) (Kliwer et al, Cell 92:73-82 (1998)). In order to examine whether the structurally-diverse compounds that activate hPXR do so by acting as ligands, three  
5 of the more potent activators representing different chemical classes were selected, dexamethasone-t-butylacetate, rifampicin, and clotrimazole, for testing in the coactivator-receptor ligand assay (CARLA). The LBDs of hPXR and mPXR1 were expressed  
10 in *E. coli* as fusion proteins with glutathione-S-transferase (GST), and SRC1.14 was synthesized *in vitro* in the presence of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine. As shown in Fig. 4C, dexamethasone-t-butylacetate, rifampicin and clotrimazole each  
15 promoted the interaction of [<sup>35</sup>S]SRC1.14 with GST-hPXR. Consistent with the results of the transfection studies, dexamethasone-t-butylacetate induced an efficient interaction between GST-mPXR1 and [<sup>35</sup>S]SRC1.14 whereas rifampicin and clotrimazole  
20 did not (Fig. 4C). Taken together, these data indicate that structurally-divergent compounds can serve as hPXR ligands, and that the human and mouse homologs of PXR differ significantly in terms of their ligand binding properties.

25

#### EXAMPLE 4

##### Transfection Assay

Plasmids: GAL4-hPXR chimera and UAS-tk-SPAP reporters. The GAL4-hPXR expression constructs contain the translation initiation sequence and  
30 amino acids 1 to 147 of the yeast *S. crevisiae* transcription factor GAL4 in the pSG5 expression

vector (Statagene). Amino acids 108 to 434 of hPXR are amplified by polymerase chain reaction (PCR) using vent polymerase (New England Biolabs) and inserted C-terminal to the GAL4 sequences. The UAS-  
5 tk-SPAP reporter contains 5 copies of the GAL4 binding site upstream of the tk promoter and the CAT gene (Berger et al, Gene 66:1 (1988)).

Transfection assay: SPAP reporter. CV-1 cells are plated in DME medium supplemented with 10%  
10 delipidated fetal calf serum at a density of  $2.4 \times 10^4$  cells per well in a 96-well plate (Costar) 16-24 h before transfection. In general, 8.0 ng of reporter plasmid, 25.0 ng of  $\beta$ -galactosidase expression  
vector (pCH110, Pharmacia), and 2.0 ng of GAL4-hPXR  
15 expression vector are mixed with carrier DNA (pBluescript, Stratagene) to a total of 80 ng of DNA per well in a volume of 10ml optiMEM I medium (Life Technologies). To this, a second mix, containing  
9.3 ml optiMEM I medium and 0.7 ml of  
20 LIPOFECTAMINE<sup>TM</sup> (Life Technologies), is added. After 30 min., an additional 80ml of optiMEM I medium are added and the combined mix is then applied to the cells. Sixteen hours later, the medium is changed to DME medium supplemented with  
25 10% delipidated and heat inactivated fetal calf serum and the test compound at a concentration of  $10^{-5}$ M. After incubation for 24 h, SPAP activity and  $\beta$ -galactosidase activity are measured by directly adding to the medium 200ml substrate mix (16mM  
30 o-nitrophenyl  $\beta$ -D-galactopyranoside (Sigma), 120mM fluorescein diphosphate (Molecular Probes), 0.16% Triton X-100, 160mM diethanolamine pH9, 44.8mM NaCl,

and 0.8mM MgCl<sub>2</sub>). Alternatively, alkaline phosphatase and  $\beta$ -galactosidase activities are measured separately using standard protocols. Briefly, cells are lysed by adding 25ml 0.5% Triton X-100 to the supernatant. To 40ml cell lysate, 200ml  $\beta$ -galactosidase substrate reagent (36mM o-nitrophenyl  $\beta$ -D-galactopyranoside, 1.25mM MgCl<sub>2</sub>, 2.8mM NaCl, 4.4M  $\beta$ -mercaptoethanol) or 200ml alkaline phosphatase substrate reagent (2.5mM p-nitrophenyl phosphate, 0.5mM MgCl<sub>2</sub>, 20mM NaCl, 1 M diethanolamine pH 9.85) are added and incubated for 1 h. Alkaline phosphatase activity is expressed as fold activation relative to that observed with vehicle alone (normalized to  $\beta$ -galactosidase activity which serves as internal control standard for transfection efficiency).

#### EXAMPLE 5

##### Synthesis of [<sup>3</sup>H]GW-485801

- (i) The Preparation of [<sup>3</sup>H]3,5-Ditertbutyl-4-hydroxy benzaldehyde.

3,5-Ditertbutyl-4-hydroxy benzaldehyde, 5 mg (20.6  $\mu$ mol) and Crabtree catalyst, 7.5 mg (9.3  $\mu$ mol), were dissolved in 2 ml dichloromethane and stirred under 10 Ci tritium gas for 5 hours. The solution was then evaporated to dryness, and labile tritium was removed by repeated evaporations from methanol. The residue was redissolved in methanol, 10 ml, counted and analyzed.

Yield = 800 mCi.

Radiochemical purity by TLC on silica in hexane:ethyl acetate (80:20) was approximately 50%.

The crude material was evaporated to 1 ml and purified by preparative plate chromatography on a single 500  $\mu$ m silica plate, eluting in hexane:ethyl acetate (85:15). The plates were viewed under UV, the band corresponding to required aldehyde was collected and the product extracted into ethyl acetate. This was evaporated to dryness and redissolved in dichloromethane, counted and analyzed.

Yield = 370 mCi.

TLC as above showed a singly labelled, specific activity 23 Ci/mmol.

15 (ii) The Preparation of [ $^3$ H]GW-485801

The product from (i) above (370 mCi at 23 Ci/mmol, 16  $\mu$ mol) was evaporated to dryness, redissolved in THF, 1 ml, and cooled in an ice bath with stirring. 1M Titanium (IV) chloride in toluene, 55  $\mu$ l, 55  $\mu$ mol, was added, immediate yellow color formed. Tetraethyl methylenediphosphonate, 75  $\mu$ l, of a THF solution at 110 mg/ml, 28.6  $\mu$ mol, was added, followed by N-methyl morpholine, 8.1  $\mu$ l, 7.5 mg, 74  $\mu$ mol. This caused a deep blue color. The solution was then stirred at room temperature for 4 hours.

TLC analysis on silica in ethyl acetate:methanol (90:10) showed approximately 60% of the radioactivity to correspond to inactive GW-485801.

(iii) The Purification of [<sup>3</sup>H]GW-485801

The crude product was purified by preparative plate chromatography on 2 x 1mm silica plates, eluting in ethyl acetate:methanol (90:10). The plates were viewed under UV, the band corresponding to required product was collected and the product extracted into ethyl acetate:methanol (90:10). This was evaporated to dryness and redissolved in nitrogen-flushed ethanol, 30 ml. This was a yellow solution.

Yield = 180 mCi.

(iv) The Analysis of [<sup>3</sup>H]GW-485801

The purified product resulting from (iii) was analyzed by TLC, HPLC, mass spectroscopy and T-NMR.

TLC showed a radiochemical purity of 99%.

HPLC showed a radiochemical purity of 98.9%.

In both of the above systems, the radioactive peak co-eluted with inactive GW-485801.

Mass spectroscopy showed a specific activity of 23 Ci/mmol, the isotope distribution being 18.4% unlabelled, 81.6% 1 x <sup>3</sup>H. The spectrum of the radioactive material was consistent with that of the inactive GW-485801.

T-NMR showed a single labelling position (peak split into four signals by coupling to the phosphorus atoms) corresponding to labelling in the vinylic position of GW-485801. This corresponds to labelling in the aldehyde-H in the precursor.

A portion of the material was diluted to 1 mCi/ml with nitrogen-flushed ethanol and dispensed as 1 X 2 mCi pack. The remainder was stored at ~20°C (approximately 170 mCi).

## EXAMPLE 6

### Biotin-His6-PXR/RXR $\alpha$ Protein

The coding sequence representing amino acids 130-434 of human PXR (Genbank AF061056) was subcloned into the pRSETa expression vector (Invitrogen). Sequence encoding a polyhistidine tag derived from an N-terminal PCR primer (MKKGHHHHHHG) (SEQ ID NO:10) was fused in-frame. The resulting encoded His6-PXR sequence was as follows:

10 MKKGHHHHHHGSERTGTQPLGVQGLTEEQRMMIRELMDAQMKTFDTTFSHK  
NFRLPGLVSSGCELPESLQAPSREEAAKWSQVRKDLCSLKVSLQLRGEDGSV  
WNYKPPADSGGKEIFSLLPHMADMSTYMFKGIISFAKVISYFRDLPIEDQIS  
LLKGAAFELCQLRFNTVFNAETGTWECGRLSYCLEDTAGGFQQLLLEPMLKF  
15 HYMLKKLQLHEEEYVLMQAISLFSPDRPGVLQHRVVDQLQEQAITLEKSYIE  
CNRPQPAHRFLFLKIMAMLTLELRSINAQHTQRLRLRIQDIHPFATPLMQELFG  
ITGS (SEQ ID NO:11).

Restriction enzymes Nde I and Hind III were used to release the cDNA fragment encoding amino acids 225-462 of RXR $\alpha$  from BB5508 (pRSETa). The fragment was ligated into the like-cut pET24a expression plasmid (Novagen). The Bgl II, Hind III fragment (contains T7 promoter, lac operator, RBS and RXR $\alpha$ ) of this construct was then cloned into the BamH I, Hind III sites (removes tetracycline resistance) of pACYC184 (BB5114). This allows for expression of RXR $\alpha$  from the T7 promoter when grown in BL21(DE3) cells and induced with IPTG. The resulting encoded RXR $\alpha$  sequence was as follows:

MKKGSANEDMPVERILEAELAVEPKTETYVEANMGLNPSSPNDPVTNICQAA  
 DKQLFTLVEWAKRIPHFSELPLDDQVILLRAGWNELLIASFHRSIAVKDGI  
 LLATGLHVHRNSAHSAGVGAI FDRVLTELVS KM RDMQMDKTELGCLRAIVLF  
 5 NPDSKGLSNPAEVEALREKVYASLEAYCKHKYPEQPGRFAKLLLRPALRSI  
 GLKCLEHLFFFKLIGDTPIDTFLMEMLEAPHQMT (SEQ ID NO:12).

The His6-PXR/pRSETa and RXR $\alpha$ /pACYC184 plasmids  
 were cotransformed into the BL21(DE3) *E. coli*  
 10 strain. One-liter shake flask liquid cultures  
 containing standard Luria-Bertani (LB) broth with  
 0.05 mg/ml Ampicillin and 0.05 mg/ml Chloramphenicol  
 were inoculated and grown at 22°C for 24 hours. The  
 cells were induced with 0.05 mM IPTG for 4-6 hours  
 15 at 22°C then the cells were harvested by  
 centrifugation (20 minutes, 3500 g, 4°C). The cell  
 pellet was stored at -80°C. The cell pellet was  
 resuspended in 250 ml Buffer A (50 mM Tris-Cl pH8.0,  
 250 mM NaCl, 50 mM imidazole pH7.5). Cells were  
 20 sonicated for 3-5 minutes on ice and the cell debris  
 was removed by centrifugation (45 minutes, 20,000g,  
 4°C). The cleared supernatant was filtered through a  
 0.45  $\mu$ m filter and loaded on to a 50 ml ProBond [Ni<sup>++</sup>  
 charged] chelation resin (Invitrogen). After  
 25 washing to baseline with Buffer A, the column was  
 washed with Buffer A containing 125 mM imidazole pH  
 7.5. The His6-PXR/RXR $\alpha$  complex was eluted from the  
 column using Buffer A with 300 mM imidazole pH 7.5.  
 Column fractions were pooled and concentrated using  
 30 Centri-prep 30K (Amicon) units. The protein was  
 subjected to size exclusion, using a column (26 mm X  
 90 cm) packed with Sepharose S-75 resin (Pharmacia)

pre-equilibrated with 20mM Tris-Cl pH 8.0, 200 mM NaCl, 5 mM DTT, 2.5 mM EDTA, pH 8.0. Column fractions were pooled and concentrated as before. The purified His6-PXR/RXR $\alpha$  was buffer exchanged by gel filtration into PBS, resulting in an average total molar protein concentration of 45mM. A five-fold total molar excess of NHS-LC-Biotin (Pierce) was added to this protein mixture in a minimal volume of PBS. This solution was incubated with gentle mixing for 60 minutes at ambient temperature, approximately 23°C. The biotinylation modification reaction was stopped by the addition of a 2000x molar excess of Tris-HCl, pH 8. The biotin-His6-PXR/RXR $\alpha$  was dialyzed at 4°C against 3 buffer changes, each of at least 50 volumes, TBS pH 8 containing 5mM DTT, 2mM EDTA and 2% sucrose. The biotin-His6-PXR/RXR $\alpha$  was subjected to mass spectrometric analysis to reveal the extent of modification by the reagent. The biotinylated protein solution was frozen and stored at -80°C.

#### EXAMPLE 7

##### PXR Scintillation Proximity Assay (SPA)

Streptavidin-PVT SPA beads (AmershamPharmacia cat # RPNQ0007) were resuspended in assay buffer (50 mM Tris HCl pH 8.0, 50 mM KCl, 1 mM DTT, 0.1 mg/ml essentially fatty acid free bovine serum albumin) at 0.5 mg/ml. Biotin-His6-PXR/RXR $\alpha$  was added to the beads to a final concentration of 50 nM. The receptors were allowed to couple to the SPA beads for thirty minutes at room temperature. The

uncoupled receptor was removed by centrifuging the SPA beads at 3000rpm for 5 minutes in a swinging bucket rotor of a Rupp & Bowman Silencer centrifuge. The receptor coated SPA beads were then resuspended in assay buffer to 3.3 mg/ml. 100 µg (30 µL) of receptor coated SPA beads were added to each well of a 96-well Optiplate (Packard cat # 6005190). Each well also contained [<sup>3</sup>H]GW-485801 at final concentrations ranging from 0.5 nM to 800 nM. Non-specific binding was determined by addition of 10 µM clotrimazole. The total volume in each well was 100 µL. The plates were sealed with TopSealA (Packard cat # 6005185) and agitated momentarily to ensure complete mixing. The plates were then allowed to incubate at room temperature until equilibrium was obtained. The plates were then counted on a TopCount liquid scintillation counter (Packard) using a protocol optimized for <sup>3</sup>H PVT SPA. Triplicate samples in the absence (T samples) or presence (NS samples) of clotrimazole were averaged and specific binding was calculated using the equation:

$$\text{specific binding} = T - NS$$

Plots of specific binding vs concentration of [<sup>3</sup>H]GW-485801 were generated (Fig. 6). K<sub>d</sub> values were determined using non-linear regression when the data were fit to the equation of a rectangular hyperbola. Test compounds were dissolved in DMSO at 10 mM and diluted 1:10 in DMSO before serially diluting in assay buffer. Compounds were typically tested at

concentrations ranging from 100 $\mu$ M to 0.3nM.

Streptavidin-PVT SPA beads (AmershamPharmacia cat # RPNQ0007) were resuspended in assay buffer (50 mM

Tris HCl pH 8.0, 50 mM KCl, 1 mM DTT, 0.1 mg/ml

5 essentially fatty acid free bovine serum albumin) at

0.5 mg/ml. Biotin-His6-PXR/RXR $\alpha$  was added to the

beads to a final concentration of 50 nM. The

receptors were allowed to couple to the SPA beads

for thirty minutes at room temperature. The

10 uncoupled receptor was removed by centrifuging the

SPA beads at 3000rpm for 5 minutes in a swinging

bucket rotor of a Rupp & Bowman Silencer centrifuge.

The receptor coated SPA beads were then resuspended

in assay buffer to 3.3 mg/ml. 100  $\mu$ g (30  $\mu$ L) of

15 receptor coated SPA beads was added to each well of

a 96-well Optiplate (Packard cat # 6005190). Each

well also contained [ $^3$ H]GW-485801 at a final

concentration of 25 nM and test compound or an equal

volume of assay buffer. Non-specific binding was

20 determined by addition of 10  $\mu$ M clotrimazole. The

total volume in each well was 100  $\mu$ L. The plates

were sealed with TopSealA (Packard cat # 6005185)

and agitated momentarily to ensure complete mixing.

The plates were then allowed to incubate at room

25 temperature until equilibrium was obtained,

approximately 1.5 hours. The plates were then

counted on a TopCount liquid scintillation counter

(Packard) using a protocol optimized for  $^3$ H PVT SPA

and programmed to correct for color quenching.

30 Values for "% [ $^3$ H]GW-485801 Bound" were calculated

using the following equation:

$$\% \text{ } [^3\text{H}]\text{GW-485801 Bound} = 100 * [(C_{\text{DPM}} - \text{NS}_{\text{DPM}}) / (T_{\text{DPM}} - \text{NS}_{\text{DPM}})]$$

where  $C_{\text{DPM}}$  is the DPM value from a well containing a  
 5 test compound,  $\text{NS}_{\text{DPM}}$  is the average of the DPM values  
 from the "non-specific" wells which contained 10  $\mu\text{M}$   
 clotrimazole,  $T_{\text{DPM}}$  is the average of the DPM values  
 from the "total" wells which contained no added  
 compounds. Graphs of  $\% [^3\text{H}]\text{GW-485801 Bound}$  vs  
 10 concentration were generated for each test compound  
 and  $\text{IC}_{50}$  values were determined using non-linear  
 regression (see Table 1).

Table 1

Compound	$\text{IC}_{50}$ ( $\mu\text{M}$ )
GW-485801	0.58
Clotrimazole	1.3
Rifampicin	2.4
5b-pregnane-3,20-dione	1.0

15

\* \* \*

All documents cited above are hereby  
 incorporated in their entirety by reference.

One skilled in the art will appreciate from a  
 reading of this disclosure that various changes in  
 20 form and detail can be made without departing from  
 the true scope of the invention.

WHAT IS CLAIMED IS:

1. An isolated human nuclear receptor that binds to a cytochrome P-450 monooxygenase promoter, or a DNA binding or ligand binding domain thereof.

2. The receptor according to claim 1 wherein the promoter is a cytochrome P-450 monooxygenase 3A4 (CYP3A4) promoter.

3. The receptor according to claim 2 wherein said receptor is hPXR.

4. An isolated human nuclear receptor having the amino acid sequence given Figure 1, or a fragment thereof, of at least 30 consecutive amino acids.

5. A fusion protein comprising a DNA binding or ligand binding domain of hPXR and a non-hPXR-derived sequence.

6. An isolated nucleic acid comprising a sequence encoding the receptor of claim 1 or 4 or the fusion protein of claim 5.

7. A construct comprising the nucleic acid of claim 6 and a vector.

8. A host cell comprising the construct of claim 7.

9. A method of making the receptor of claim 3, or fragment thereof, comprising:

culturing a host cell containing an expression construct comprising a sequence encoding said receptor, or fragment thereof, operably linked to a promoter, under conditions such that said receptor, or fragment thereof, is produced, and

isolating said receptor, or fragment thereof.

10. A method of screening a test compound for its ability to induce CYP3A4 gene expression comprising

i) contacting said test compound with the ligand binding domain of hPXR,

ii) determining whether said test compound binds to said ligand binding domain, wherein binding of the test compound to said ligand binding domain is indicative of a compound that induces CYP3A4 gene expression.

11. A method of screening a test compound for its ability to activate or inhibit hPXR comprising:

i) preparing an expression vector comprising a sequence encoding a DNA binding domain and a hPXR ligand binding domain;

ii) preparing a reporter construct comprising a DNA binding site recognized by said DNA binding domain operably linked to a reporter gene,

iii) introducing said expression vector and said reporter construct into compatible host cells,

iv) incubating said cells resulting from step (iii) with said test compound, and

v) determining the level of expression of said reporter gene,

wherein enhancement of expression of said reporter gene in the presence of said test compound indicates that said test compound can activate hPXR, and

wherein inhibition of expression of said reporter gene in the presence of said test compound indicates that said test compound can inhibit hPXR.

12. A compound that induces CYP3A4 identified by the method of claim 10.

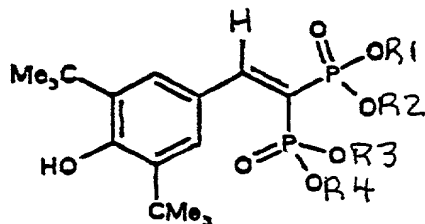
13. A compound that activates hPXR identified by the method of claim 11.

14. A method of modulating function of a cell mediated by PXR comprising contacting said cell with a compound identified using the method of claim 11 that activates PXR under conditions such that said activation is effected and said function is thereby modulated.

15. A method of modulating function of a cell mediated by PXR comprising contacting said cell with a compound identified using the method of claim 11 that inhibits PXR under conditions such that said inhibition is

effected and said function is thereby modulated.

16. The method according to claim 14 or 15 wherein said compound is of formula I:



wherein R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> and R<sub>4</sub> are, independently, C<sub>1</sub>-C<sub>6</sub>alkyl, linear or branched.

17. The method according to claim 14 or 15 wherein said cell is a cultured cell.

18. The method according to claim 14 or 15 wherein said cell is present in a tissue.

19. The method according to claim 14 or 15 wherein said cell is present in an animal.

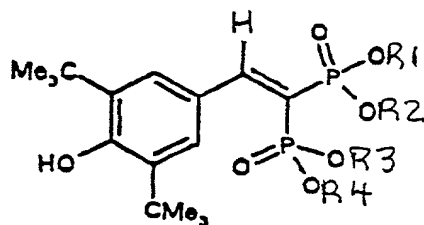
20. A method for associating a particular disease or condition with modulation of PXR comprising

contacting a compound that binds to PXR specifically with PXR present in a cell under conditions such that said binding is effected

and a functional activity of said cell mediated by PXR is thereby modulated,

detecting said modulation of said functional activity and associating said modulation of said functional activity with a disease or condition and thereby associating said disease or condition with modulation of PXR.

21. The method according to claim 20 wherein said compound is of formula I:



wherein R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> and R<sub>4</sub> are, independently, C<sub>1</sub>-C<sub>6</sub>alkyl, linear or branched.

22. The method according to claim 21 wherein said compound is GW-485801.

23. A method of preventing or treating a disease or condition that has been associated with modulation PXR by the method of claim 20, comprising administering to a patient in need thereof a therapeutically effective amount of an agent that modulates the activity of PXR so that said prevention or treatment is effected.

24. The method according to claim 23  
wherein said agent is GW-485801.

ABSTRACT OF THE DISCLOSURE

5 The present invention relates to a novel human  
orphan nuclear receptor that binds to a cytochrome  
P-450 monooxygenase (CYP) promoter and that is  
activated by compounds that induce CYP gene  
expression. The invention further relates to  
nucleic acid sequences encoding such a receptor, to  
methods of making the receptor and to methods of  
using the receptor and nucleic acid sequences  
10 encoding same. The invention also relates to non-  
human animals transformed to express the human  
receptor and to methods of using such animals to  
screen compounds for drug interactions and  
toxicities.

A

```

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61  GACGGGAAGAGGAAGCACTGCCTTTACTTCAGTGGGAATCTCGGCTCAGCCTGCAAGCC
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    L E V R P K E S W N H A D F V H C E D 19
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Figure 1A

**B**

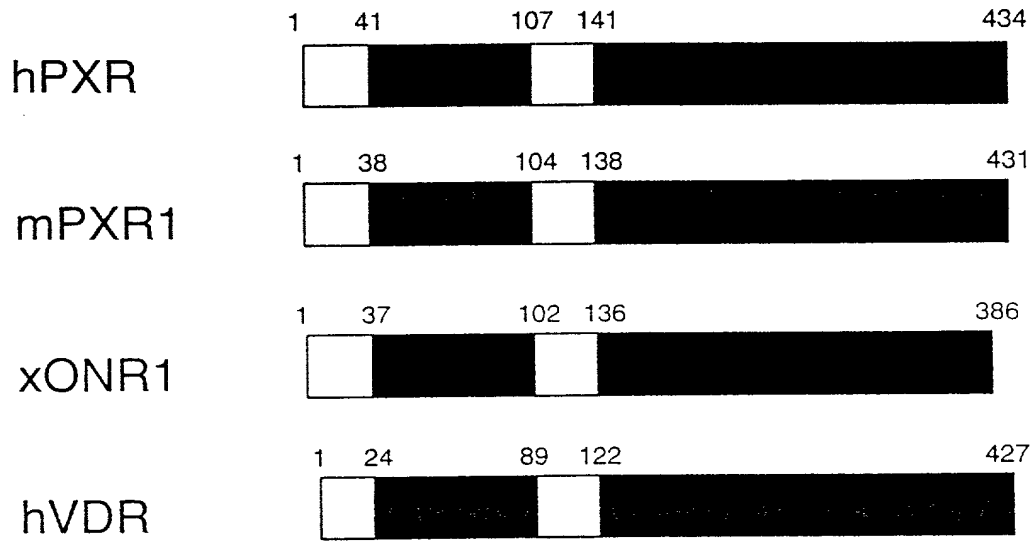


Figure 1B

**C**

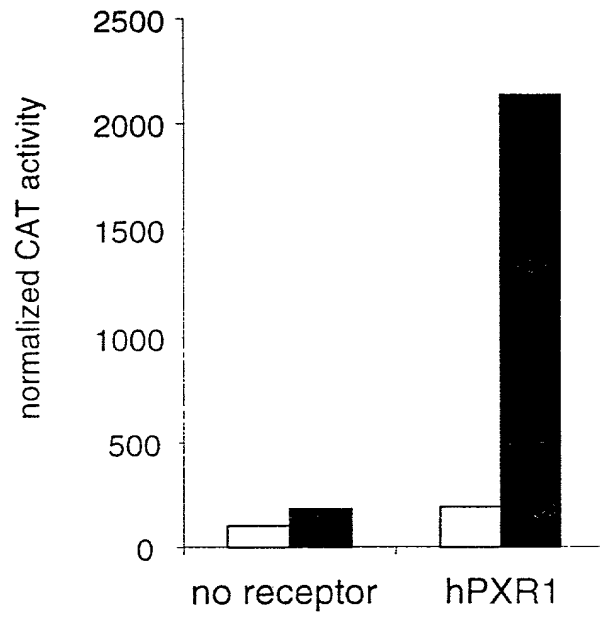


Figure 1C

**D**

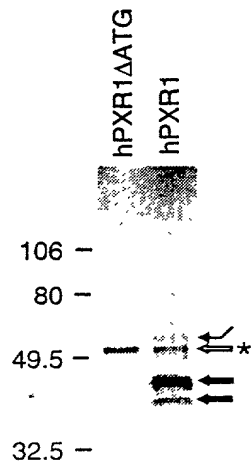


Figure 1D

009200 569200

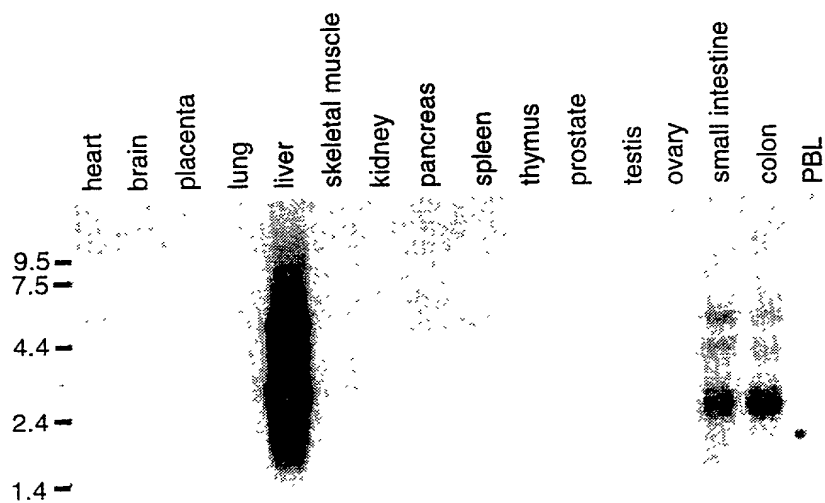


Figure 2

**A**

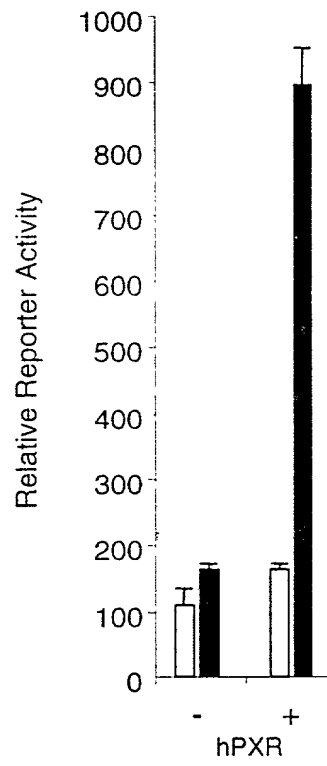
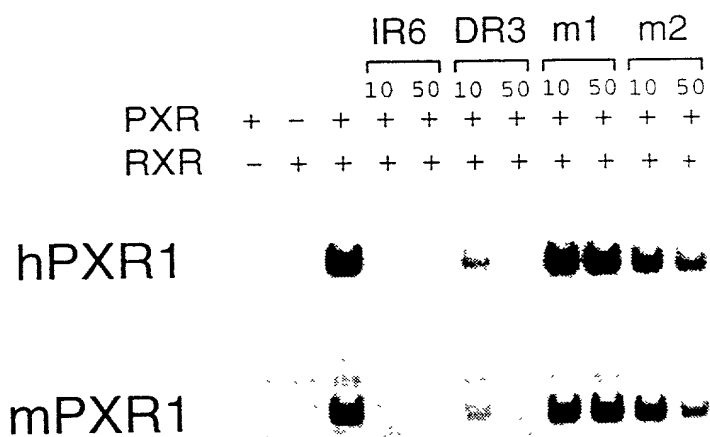


Figure 3A

**B**

CYP3A4 IR6	ata	<b>TGAACT</b>	caaagg	<b>AGGTCA</b>	gtg
		<-----		----->	
CYP3A4 IR6 m1	ata	<b>TGTTCT</b>	caaagg	<b>AGAACA</b>	gtg
		<-xx--		--xx->	
CYP3A4 IR6 m2	ata	<b>ACAACT</b>	caaagg	<b>AGGTCA</b>	gtg
		xx----		----->	
CYP3A1 DR3	aga	<b>TGAACT</b>	tca	<b>TGAACT</b>	gtc
		<-----		<-----	

**C**



Figures 3B and 3C

**A**

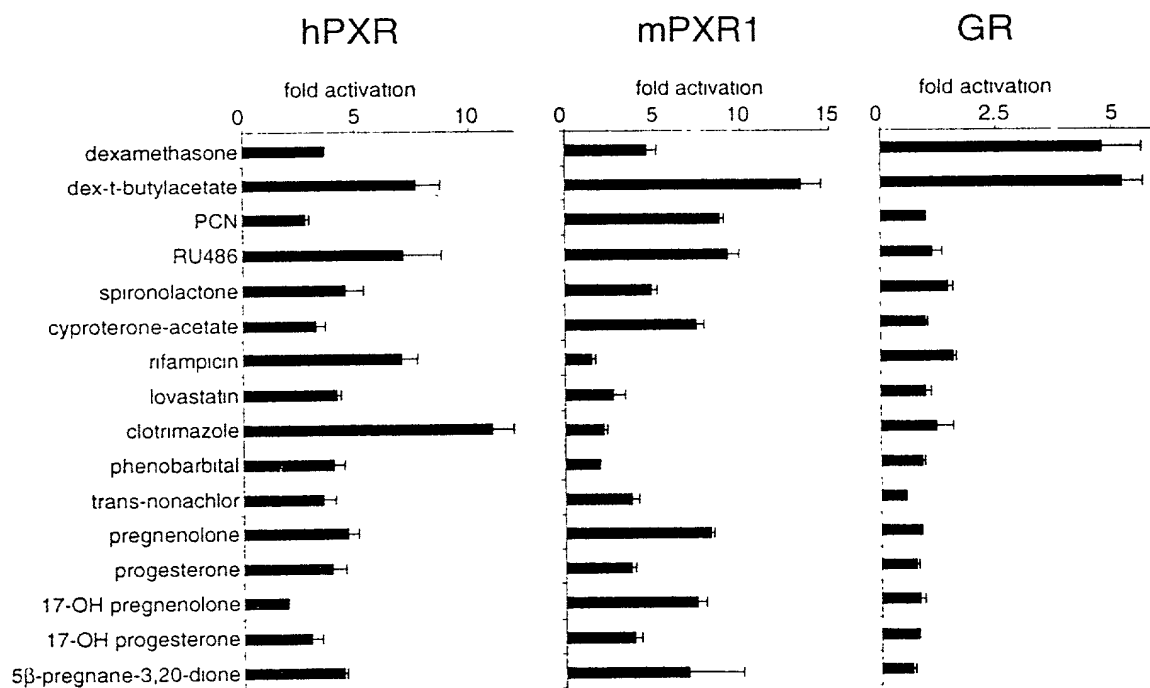


Figure 4A

**B**

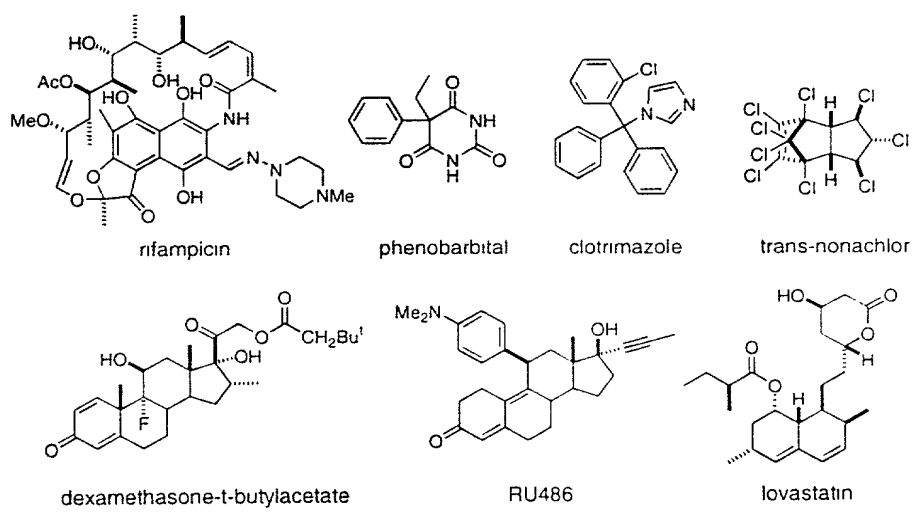


Figure 4B

C

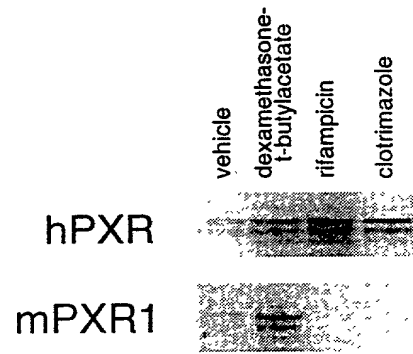
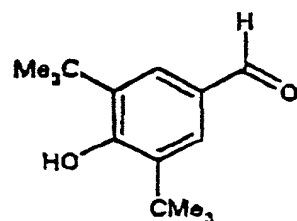


Figure 4C

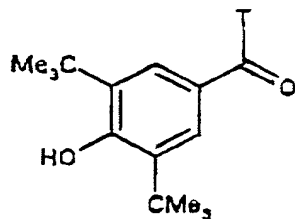
The Preparation of [<sup>3</sup>H]GW-485801

Figure 5

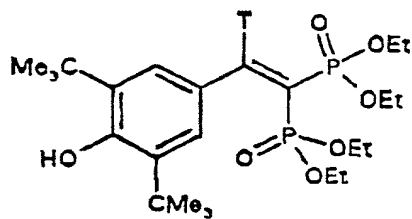
1. REACTION SCHEME



Crabtree catalyst, dichloromethane  
Tritium gas



Titanium chloride,  
tetraethyl methylenediphosphonate,  
methyl morpholine

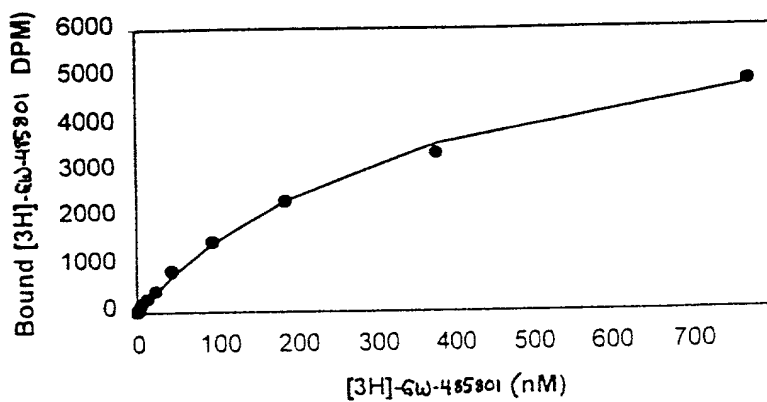


[<sup>3</sup>H]GW-485801

009280-9280

Figure 6

$K_d = 370 \text{ nM}$



# SEQUENCE LISTING

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 JONES, Stacey A.  
 WILLSON, Timothy M.

<120> AN ORPHAN NUCLEAR RECEPTOR

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Glu Leu Pro Glu Ser Leu Gln Ala Pro Ser Arg Glu Glu Ala Ala Lys  
65 70 75 80  
Trp Ser Gln Val Arg Lys Asp Leu Cys Ser Leu Lys Val Ser Leu Gln  
85 90 95  
Leu Arg Gly Glu Asp Gly Ser Val Trp Asn Tyr Lys Pro Pro Ala Asp  
100 105 110  
Ser Gly Gly Lys Glu Ile Phe Ser Leu Leu Pro His Met Ala Asp Met  
115 120 125  
Ser Thr Tyr Met Phe Lys Gly Ile Ile Ser Phe Ala Lys Val Ile Ser  
130 135 140  
Tyr Phe Arg Asp Leu Pro Ile Glu Asp Gln Ile Ser Leu Leu Lys Gly  
145 150 155 160  
Ala Ala Phe Glu Leu Cys Gln Leu Arg Phe Asn Thr Val Phe Asn Ala  
165 170 175  
Glu Thr Gly Thr Trp Glu Cys Gly Arg Leu Ser Tyr Cys Leu Glu Asp  
180 185 190  
Thr Ala Gly Gly Phe Gln Gln Leu Leu Leu Glu Pro Met Leu Lys Phe  
195 200 205  
His Tyr Met Leu Lys Lys Leu Gln Leu His Glu Glu Glu Tyr Val Leu  
210 215 220  
Met Gln Ala Ile Ser Leu Phe Ser Pro Asp Arg Pro Gly Val Leu Gln  
225 230 235 240  
His Arg Val Val Asp Gln Leu Gln Glu Gln Phe Ala Ile Thr Leu Lys  
245 250 255  
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 Glu Glu Val Gly Gly Pro Gln Ile Cys Arg Val Cys Gly Asp Lys Ala  
 35 40 45  
 Thr Gly Tyr His Phe Asn Val Met Thr Cys Glu Gly Cys Lys Gly Phe



